Chemokine-idiotype fusion DNA vaccines are potentiated by bivalency and xenogeneic sequences

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V regions of monoclonal Ig express an exquisite B-cell tumor–specific antigen called idiotype (Id). Id is a weak antigen and it is important to improve immunogenicity of Id vaccines. Chemokine receptors are expressed on antigen-presenting cells (APCs) and are promising targets for Id vaccines. Here we compare monomeric and dimeric forms of MIP-1α and RANTES that target Id to APCs in a mouse B lymphoma (A20) and a multiple myeloma model (MOPC315). MIP-1α was more potent than RANTES. The dimeric proteins were more potent than monomeric equivalents in short-term assays. When delivered in vivo by intramuscular injection of plasmids followed by electroporation, dimeric proteins efficiently primed APCs in draining lymph nodes for activation and proliferation of Id-specific CD4+ T cells. Good anti-Id antibody responses were obtained, and mice immunized only once were 60% to 80% protected in both tumor models. CD8+ T cells contributed to the protection. Antibody responses and tumor protection were reduced when the human Ig hinge + C_H3 dimerization motif was replaced with syngeneic mouse counterparts, indicating that tumor-protective responses were dependent on xenogeneic sequences. The results suggest that bivalency and foreign sequences combine to increase the efficiency of chemokine-Id DNA vaccines. (Blood. 2007;110:1797-1805)

Introduction

B lymphomas and multiple myelomas (MMs) produce monoclonal Ig, the V regions of which can serve as targets for tumor-specific immune responses.[1,2] The V region antigenic determinants (idiotype [Id]) arise as a consequence of clone-specific V(D)J rearrangements and somatic mutations. Id can be directly recognized in 3 ways: (1) by Ab,[3] (2) by CD4+,[4] and (3) by CD8+ T cells.[5] Id-specific CD4+ and CD8+ T cells recognize V region–derived Id peptides presented on MHC class II and I molecules, respectively. While B-lymphoma cells display all 3 types of Id targets on their cell surface, MM cells often express little surface Ig and MHC class II. Nevertheless, Id-specific CD4+ T cells can confer protection against MHC class II–negative MM cells because secreted myeloma protein is processed and presented by tumor-infiltrating antigen-presenting cells (APCs) to Id-specific CD4+ T cells.[6-8]

Immunization with myeloma protein in complete Freund adjuvant (CFA) induced an Id-specific resistance against tumor challenge in mice,[1] a finding that has been confirmed and extended in a number of experimental mouse studies. Id vaccination has entered clinical trials, with more promising results in B-cell lymphoma[9] than in MM[10] patients. However, Id is a weak antigen and a number of innovative strategies have been used to increase its immunogenicity. Id-pulsed dendritic cells (DCs)[11] are promising, but suffer from labor-intensive manufacture. Attractive alternatives are to fuse Ig, Fab, or single-chain fragment variable (scFv) to GM-CSF,[12] chemokines,[13-15] CD40Ligand,[16] tetanus toxin fragment C,[17] and Il-1β[18] and to deliver these as protein[12,13,16-18] or DNA[13,17,18] vaccines. Such immunizations have generated tumor-protective responses by mechanisms that are not fully elucidated, but that most likely include targeting of Id to APCs, APC maturation, or both.

Chemokines control migration of specific leukocyte populations during inflammatory responses, hematopoiesis, and routine immune surveillance. RANTES (regulated upon activation normal T-cell expressed, CCL5) and MIP-1α (macrophage inflammatory protein 1α, CCL3) are inflammatory chemokines. They both have high affinity for CCR1 and CCR5 expressed on T cells, monocytes, natural killer cells, and DCs.

MIP-1α and RANTES self-associate to form high-molecular-mass aggregates.[19,20] However, the activation state of chemokine monomers versus oligomers has been a disputed field.[21-23] It has been shown that chemokine receptors initiate their ligand-induced signaling cascades by receptor dimerization.[24-26] Monomeric variants of RANTES and MIP-1β retain full activity in vitro, but are devoid of activity in vivo, suggesting that these chemokines require oligomerization to recruit cells in vivo.[27] It has been proposed that although chemokines are able to interact with receptors as monomers,[21] glycosaminoglycan-induced oligomerization of chemokines can achieve higher order oligomers in vivo, and they may interact with receptors differently as dimers or tetramers, or heparin-chemokine complexes.[28] Oligomerized chemokines could, in principle, transmit different signals than monomeric chemokines to receptor-bearing leukocytes[29,30] or via cell surface GAGs on the endothelium.

Id containing xenogeneic (human) constant region sequences showed increased immunogenicity and were more potent at inducing anti-Id antibodies than native Id.[12,31] This was proposed to be due to the introduction of foreign helper epitopes. The critical

Submitted June 30, 2006; accepted May 29, 2007. Prepublished online as *Blood* First Edition paper, May 31, 2007; DOI 10.1182/blood-2006-06-032938.

The online version of this article contains a data supplement.

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importance of $CD4^+$ T-cell help for the maintenance and expansion of memory B cells has recently been further elucidated.[32]

We have here produced dimeric Id vaccines (vaccibodies), each chain consisting of 3 structures fused in the following order: (1) chemokine (MIP-1α or RANTES), (2) a dimerization domain, either mouse or human Ig hinge + C_H3, (3) scFv (from A20 lymphoma or MOPC315 myeloma). These bivalent (chemokine-Id)$_2$ molecules were compared with monovalent chemokine-Id equivalents.

Materials and methods

Mice, cell lines, and antibodies

All animal experiments were approved by the Norwegian Animal Research Authority. Six- to 10-week-old BALB/c mice and $\lambda2^{315}$-specific MHC class II ($I\text{-}E^d$)–restricted TCR-transgenic mice on a severe combined immunodeficient (SCID) background[33] were bred by Taconic (Ry, Denmark). The HEK293E,[34] NS0, MOPC315 (IgA, λ2),[1] and P388D1 cell lines were purchased from ATCC (Manassas, VA). The MOPC315.4 subline used herein has been selected for good growth in vivo and in vitro.[35] The BALB/c B cell lymphoma A20 (IgG2a, κ) cell line,[36] subline A20.1.11, was kindly provided by Prof S. Buus (University of Copenhagen, Denmark). The 91-101 $\lambda2^{315}$-specific, $I\text{-}E^d$–restricted $CD4^+$ T-cell clone 7A10B2 has been described previously.[4]

Construction of chemokine-vaccines

Antigenic and dimerization units. Construction of the antigenic units $scFv^{315}$ and $scFv^{A20}$, and the human dimerization unit consisting of h1, h4, and C_H3 exons from human γ3, have been previously described.[37] The murine dimerization unit consisting of the complete hinge and C_H3 exons from mouse γ2b were cloned from the pLNOH2-γ2b plasmid[38] using specific primers including restriction enzyme sites and linkers (Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The hinge and C_H3 gene segments were joined by polymerase chain reaction (PCR) SOEing, and then joined to the $scFv^{315}$ or $scFv^{A20}$ by a subsequent PCR SOEing before these constructs were inserted into the C cassette of the expression vector pLNOH2,[39] essentially as previously described.[37]

The targeting unit (chemokines). Four percent thioglycolate-stimulated peritoneal cells were isolated by lavage after 3 days. mRNA was extracted from the cells with oligo (dT)–coated magnetic Dynabeads (Invitrogen Dynal AS, Oslo, Norway). First-strand cDNA was synthesized and used as template for PCR amplification of chemokine genes using specific primers annealing to the ends of RANTES and MIP-1α (Document S1). The chemokine PCR products were cloned into the T-tagged site of pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's instructions. To abrogate binding of MIP-1α and RANTES to their receptors, the first cysteines (positions 10 and 11, respectively) were exchanged with serines[40] by Quick Change PCR (Document S1). After sequencing, the pGEM-T chemokine vectors were digested with *Bsm*I and *Bsi*WI, and the chemokine genes were subcloned into the V cassette of the pLNOH2 vector that in the C cassette already contained either the mIgG2b(hinge-$C_\gamma3$)-$scFv^{315}$ (or $scFv^{A20}$) (generating [chemokine $mF_v^{315\text{ or }A20}]_2$) or the hIgG3(hinge-$C_\gamma3$)-$scFv^{315}$ or $scFv^{A20}$ (generating [chemokine $hF_v^{315\text{ or }A20}]_2$).[37]

To construct the monomeric MIP-1α-scFv variants, primers with spacer (NDAQAPKS)[13] overhangs (bold) were used to fuse the chemokines in frame with the antigenic scFv's by PCR and PCR SOEing (Document S1). These PCR products were digested with *Bsm*I and *Bam*HI and subcloned into an empty pLNOH2 vector resulting in (chemokine-$F_v^{315\text{ or }A20}$) constructs.

Production and purification of chemokine-scFv proteins

The pLNOH2 vector carrying different variants of chemokine-scFv genes (Figure 1), expressed from a CMV promoter, was stably transfected into NS0 cells by electroporation and cloned in 800 μg/mL G418. Since M315 myeloma protein binds DNP/TNP,[41] $scFv^{315}$-containing proteins were affinity purified from supernatant on a DNP (dinitrophenyl)-lysine-Sepharose column (Sigma, St Louis, MO). For transient expression of vaccine proteins, plasmids were transfected into HEK293E cells with lipofectamine 2000 (Invitrogen, Frederick, MD). The supernatant was harvested on day 3.

Measurement of chemokine-scFv proteins, anti-Id antibodies, and M315 myeloma protein by sandwich enzyme-linked immunosorbent assays (ELISAs)

Dimeric chemokine-scFv (vaccibody) proteins. Antimouse RANTES mAb, antimouse MIP-1α mAb (both R&D Systems, Minneapolis, MN), DNP-BSA, or Ab2.1-4 mouse mAb specific for scFv315 (ie, Id^{315}) was used as coat, while biotinylated 9A8 rat antimouse Vλ mAb (detects Fv^{315} that has a Vλ2 domain), biotinylated HP6017 mouse anti–human IgG (Fc) (Zymed Laboratories, South San Francisco, CA), or biotinylated rat anti–mouse IgG2b (BD Pharmingen, San Diego, CA) was used as detection mAb. Affinity-purified vaccibody proteins served as standards.

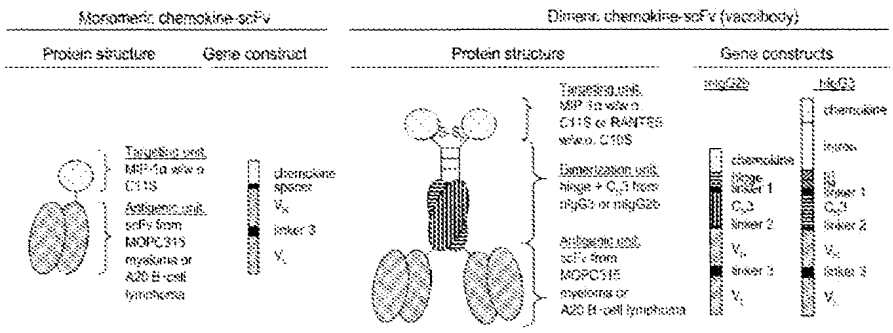

Figure 1. Monomeric and dimeric chemokine-Id fusion proteins and their genetic construction. (Left) Schematic structure of monomeric chemokine-Id fusion protein and the corresponding gene construct. The mouse MIP-1α chemokine gene, or a control construct that encodes MIP-1α with point mutations replacing the first cysteine with serine (C11S), was fused in frame with the scFv, separated by a spacer fragment (NDAQAPKS) to enable proper folding of the proteins.[13] (Right) Structure of homodimeric chemokine-Id fusion protein (vaccibody) and the corresponding gene constructs. Targeting, dimerization, and antigenic units are indicated, as are variants in the different units (see also Table S1). In the monomeric chemokine-scF_v constructs, the MIP-1α and RANTES mouse chemokine genes, or variants where the first cysteine had been destroyed, were inserted in the targeting cassette of the pLNOH2 vector. In the vaccine constructs with a dimerization unit derived from mouse IgG2b, the single hinge exon (4 cysteines that can form disulphide bonds) was fused in frame with a $G_3S_2G_3SG$ linker (linker 1) and the $C_\gamma3$ exon. A short GLGGL linker (linker 2) connects the $C_\gamma3$ exon with the antigenic scFv from either MOPC315 myeloma or the A20 B-cell lymphoma. In the constructs with a dimerization unit derived from human IgG3, there is an intronic sequence upstream of the h1 hinge exon, which is fused in frame with the h4 exon (altogether 3 cysteines that can form disulphide bonds) and linker 1 followed by the $C_\gamma3$ exon, a GLGGL sequence (linker 2) and scFv. A $(G_4S)_3$ linker (linker 3) connects the V_H and V_l in the antigenic unit.

Anti-idiotypic antibodies with specificity for Fv^{315}. Myeloma protein M315 (IgA, λ2) was used as coat and anti-Id Abs were detected by a biotinylated antimouse κ mAb (187.1 bio). Ab2.1-4 (IgG1, κ) anti-Id^{315} mAb was used as standard. To determine heavy-chain isotypes, biotinylated anti–mouse IgG1 and IgG2a (BD Pharmingen) were used as detection mAbs, and end-point titers were calculated.

Flow cytometry

BALB/c splenocytes were incubated with anti-CD11b PE and chemokine-scFv proteins. After washings, bound fusion proteins were revealed by either biotinylated HP6017 (anti-hIgG Fc) or anti-mIgG2b mAb or Ab2.1-4 mAb (anti-Id^{315}) and streptavidin PerCP. Twenty thousand cells were run on FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using CellQuestPro software (BD Biosciences, San Jose, CA).

Metabolic labeling and immunoprecipitation

NS0 cells transfected with chemokine-scFv^{315} constructs were labeled with ^{35}[S]-methionine and ^{35}[S]-cysteine (Amersham, Arlington Heights, IL) essentially as described.[47] The supernatant was incubated with anti-Id^{315} Ab2-1.4 mAb, and precipitated with magnetic beads coated with sheep anti–mouse IgG Dynabeads (Dynal). Eluted proteins were run on a 4% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Actin polymerization assay

BALB/c peritoneal macrophages (1×10^6) or immature bone marrow–derived DCs, prepared essentially as described,[42] were stained with anti-CD11b PE or anti-CD11c PE before addition of 10 ng/mL chemokine-scFv proteins. Reactions were stopped at 15, 60, 300, and 600 seconds by fixing the cells with a staining cocktail containing 8% paraformaldehyde, 1 μg/mL FITC-phalloidin, and 0.5 mg/mL L-α-lysophosphatidylcholin, followed by flow cytometric analysis. The relative increase in fluorescence over that of nonstimulated cells was plotted.

Chemotaxis

In vitro chemotaxis. Cell migration was assessed by a 48-well microchemotaxis chamber (NeuroProbe, Gaithersburg, MD) technique as described previously[43] (Document S1).

In vivo chemotaxis. Eight- to 10-week-old female BALB/c mice were injected intraperitoneally with chemokine-fusion proteins or PBS alone. Based on previous experiments,[27] the concentration of different chemokine-fusion proteins was adjusted to 1.5 μM of the chemokine moiety and 200 μL was injected. After 18 hours, mice were killed by cervical dislocation and the peritoneal cavity was flushed with a total of 10 mL RPMI medium and cells/mL determined. The frequency of CD11c$^+$ cells was determined by flow cytometry and absolute numbers were calculated.

Protein and DNA immunizations

Six- to 10-week-old BALB/c or Id (λ2^{315})–specific TCR-transgenic SCID mice were injected intraperitoneally with 100 μg chemokine-scFv proteins or anesthetized and injected with 50 μL plasmids (purified with Endofree Qiagen kit; Hilden, Germany) in both quadriceps muscles, and electroporated, as previously described.[44] Blood samples were collected at the time points indicated. Groups consisted of 7 to 10 mice; mean plus or minus SEM is given.

T-cell assays, APC assays, and flow cytometry

In vitro T-cell proliferation assay. T-cell proliferation assays were performed essentially as described previously[45] (Document S1).

In vivo detection of primed LN APCs. BALB/c mice were injected intramuscularly with 50 μg vaccibody plasmids and electroporated. Eight days later, draining (lumbar and sacral) and nondraining (mesenteric) lymph node cells were treated with collagenase and DNase,[7] irradiated (20 Gy), and 5×10^5 cells/well were incubated with 2×10^4 cloned λ2^{315}-specific 7A10B2 CD4$^+$ T cells[4] in a proliferation assay as described above. To determine CD69 expression, 10 days after vaccination of λ2^{315}-specific TCR transgenic SCID mice,[33] draining and nondraining lymph node cells were triple stained with biotinylated GB113 mAb clonotype specific for the transgenic TCR,[46] APC-conjugated anti-CD4 mAb, and PE-conjugated anti-CD69 mAb (BD Pharmingen). The biotinylated GB113 mAb was detected by streptavidin-CyChrome. To determine BrdU incorporation, DNA-vaccinated BALB/c mice were reconstituted intravenously on day 8 with 1.5×10^7 lymph node cells from λ2^{315}-specific TCR-transgenic mice.[46] On day 11, 1 mg BrdU was injected intraperitoneally into each mouse and the mice were given 1 mg/mL BrdU in the drinking water. At day 14, gated F23.1$^+$GB113$^+$ CD4$^+$ T cells in the draining and nondraining LNs were stained with APC BrdU flow kit (BD Biosciences). The cells were run on a FACSCalibur cytometer.

Tumor challenge

MOPC315 cells (1.3×10^5) or A20 cells (1.2×10^5) were injected subcutaneously in the right flank region 14 days after vaccination with protein or DNA/electroporation. Tumor growth was monitored by palpation and by use of a caliper. The mice were killed when the tumor diameter reached 15 mm. Tumor avoidance curves and statistical analyses were calculated by use of GraphPad Prism 4.0 software (San Diego, CA).

In vivo T-cell depletions

Antibody depletions started 12 days after DNA vaccination with 400 μg anti-CD8 mAb 53.6.72, anti-CD4 mAb GK1.5, anti–MHC class II mAb 14-4-4S, or isotype controls, delivered intraperitoneally. Treatments were repeated every second day for 14 days and were then reduced to once a week. T-cell subset depletion was confirmed by staining of peripheral blood leukocytes during the experiment.

Results

Dimeric and monomeric chemokine-scFv fusion proteins for Id vaccination

The monomeric mouse MIP-1α-scFv (Figure 1) was essentially constructed as previously described by Biragyn et al for other chemokines.[13] As nontargeted controls, we generated variants where we mutated the first cysteine in the chemokine to a serine, which destroys a disulphide bond and thereby the ability to bind chemokine receptors CCR1 and CCR5.[40] As scFv, we used V regions from the myeloma cell line MOPC315[1] and the B-lymphoma cell line A20[36] (Figure 1).

The dimeric equivalent of MIP-1α-scFv was constructed by modifying the recently published vaccibody structure[37] by exchanging the targeting unit, scFv specific for MHC class II, with MIP-1α. As nontargeted controls, we generated Cys-Ser variants. As dimerization unit, we used either of 2 different types. The first had a shortened hinge (h1 + h4 exons) and C_H3 of human γ3, and that has been shown to cause formation of dimers due to disulphide bonds between the monomers.[37] The drawback of such a dimerization unit is its xenogeneic and potentially immunogenic nature in mice. We therefore constructed a second, syngeneic dimerization unit composed of the complete hinge exon and C_H3 from mouse IgG2b. Antigenic units were either scFv^{A20} or scFv^{315} (Figure 1).

We similarly constructed dimeric chemokine-scFv molecules with another chemokine, RANTES, that binds CCR1, CCR3, and CCR5.[47] A number of variants were generated, including (1) the Cys-Ser mutation that destroys binding to CCR, (2) human or mouse dimerization units, and (3) scFv^{A20} or scFv^{315} as tumor antigens (Figure 1). In the case of RANTES, we made only dimeric, and not monomeric, chemokine-scFv fusion proteins.

Characterization of chemokine-scFv fusion proteins

Supernatants of transfected HEK293E cells, or affinity-purified proteins from supernatant of stably transfected NS0 cells, were used as sources of chemokine-Id fusion proteins. Proper folding was confirmed by ability to bind a panel of mAbs recognizing the chemokine moieties, the dimerization units, and the antigenic scFv^{315} in ELISA (Table S1). The ability of chemokines to bind macrophages that express relevant CCRs was shown by flow cytometry (Figure 2A; Table S1). No binding to chemokine-specific mAbs or macrophages was observed for variants where the first cysteine in the chemokines had been mutated to a serine, thus, these variants served as nontargeted (negative) controls in the experiments. Homodimerization of vaccibodies was confirmed by SDS-PAGE under nonreducing conditions. Also, monomeric chemokine-scFv had the expected size (Figure 2B).

Chemokine-scFv fusion proteins induce actin polymerization and chemotaxis

Binding of MIP1-α and RANTES to chemokine receptors is accompanied by a change in the configuration of intracellular actin in the cytoskeleton. We therefore used the F-actin polymerization assay[48] to study the biologic effect of the chemokine-scFv^{315} fusion proteins. CD11b$^+$ peritoneal macrophages or immature bone marrow–derived DCs were treated with chemokine-fusion proteins or recombinant human RANTES as a positive control. After different time points, the reactions were stopped and permeabilized cells were stained for detection of polymerized actin. Both MIP-1α-scFv and RANTES-scFv fusion proteins induced F-actin polymerization. There were only marginal differences between the constructs; however, all the dimeric fusion formats were more active than the monomer, especially with BM-DCs. Overall, the BM-DCs were more responsive than macrophages. Mutants where the first cysteine in the chemokines had been mutated were inactive (Figure 3A,B). Similar results were obtained with chemokine-fusion proteins where scFv^{315} was exchanged with scFv^{A20}.

We next tested the ability of chemokine-scFv^{315} fusion proteins to induce chemotaxis in vitro. Dose-response studies showed that the various chemokine-fusion proteins stimulated migration in a dose-dependent manner. However, while 1 nM chemokine was needed to reach the maximum level of migration for the MIP-1α fusion proteins, 10-fold more was required for the RANTES fusion proteins and rRANTES. All dimeric formats were slightly more active than the monomeric format. The difference was more pronounced with BM-DCs than macrophages. As observed for actin polymerization, BM-DCs were chemotactically more responsive than macrophages. C11S and C10S variants were inactive, demonstrating that the responses obtained with RANTES and MIP-1α fusion proteins were mediated by functional chemokine domains of the fusion proteins (Figure 3C,D). Similar results were obtained with chemokine-fusion proteins where scFv^{315} was exchanged with scFv^{A20} (Table S1).

To test the chemotactic abilities in vivo, chemokine-scFv^{315} fusion proteins were injected into the peritoneal cavity of BALB/c mice. Eighteen hours later, the peritoneal cavities were flushed and cell concentrations determined in the lavage fluid. Dimeric MIP-1α fusion proteins, either with human or mouse dimerization unit, induced a robust 5-fold increase in the number of recruited cells compared with controls (Figure 3E). Monomeric MIP-1α F$_v^{315}$ was much less effective, while dimeric RANTES fusion protein was intermediate. Variants with mutated first cysteines were ineffective. The same pattern of effectiveness, $MIP_2 > RANTES_2 > MIP_1$ was observed for recruitment of CD11c$^+$ DCs (Figure 3F).

Intact chemokine, dimeric structure, and foreign human Ig dimerization unit all contribute to induction of strong, lasting anti-Id antibody responses

Mice were injected intramuscularly with plasmids encoding different chemokine-fusion molecules that had scFv^{315} as antigenic unit. The injection site was immediately electroporated in order to increase uptake and expression of plasmids.[49] As can be seen (Figure 4), the success of inducing sustained levels of anti-Id^{315} antibodies depended on a number of factors. Targeting by MIP-1α was slightly more effective than by RANTES. Intact chemokines were required because the Cys-Ser mutants induced only delayed and low levels of antibodies. Dimeric constructs were more effective than the monomeric. A foreign human dimerization unit was much more effective than a mouse equivalent, the latter failing to induce long-lasting antibody levels. The antibody responses were antigen specific, since no anti-Id^{315} antibodies were detected when the antigenic scFv^{315} unit was replaced with scFv^{A20} (Figure 4). The antibodies were a mixture of IgG2a and IgG1 isotypes; although the IgG2a dominated (data not shown). In conclusion, dimeric MIP-1α (or RANTES) chemokine-scFv with human dimerization units were superior at inducing sustained anti-Id responses after a single DNA immunization.

CD4$^+$ T cells are stimulated by chemokine-fusion vaccines in vitro

To investigate whether the antigen carried by the chemokine-fusion proteins was efficiently processed and presented by APCs to CD4$^+$

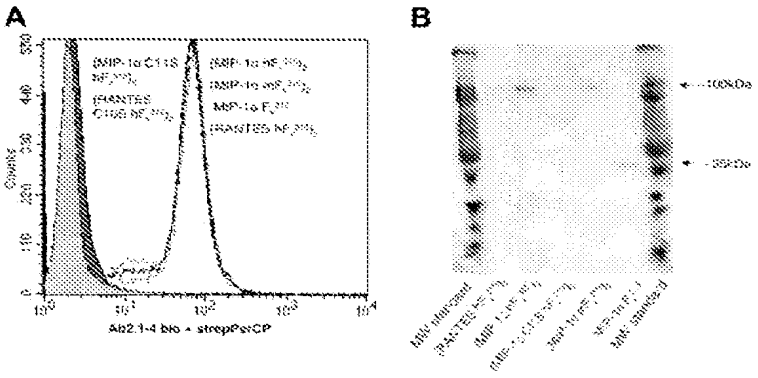

Figure 2. Characterization of chemokine-fusion proteins. (A) Staining of BALB/c CD11b$^+$ splenocytes with vaccine proteins. Both MIP-1α and RANTES maintain binding to CD11b$^+$ cells in all 3 fusion protein formats, while the mutation of the first cysteine to serine in MIP-1α and RANTES abrogates binding to CD11b$^+$ cells. Bound proteins were detected by fluorochrome-labeled HP6017 mAb that binds hIgG Fc. (B) Vaccibodies with the different targeting and dimerization units all form functional homodimers of approximately 100 kDa, while the monomeric chemokine-scFv protein has a molecular weight of approximately 35 kDa.

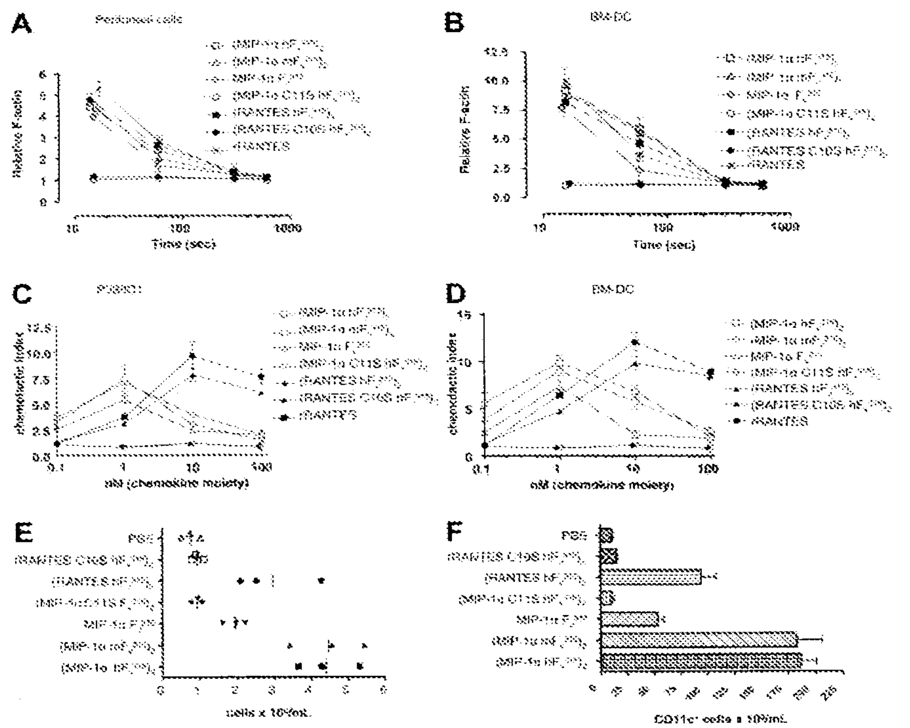

Figure 3. Chemokine-scFv fusion proteins induce F-actin polymerization and chemotaxis. (A,B) F-actin polymerization in BALB/c peritoneal macrophages (A) or immature bone marrow–derived DCs (B) after stimulation with 10 ng/mL chemokine-fusion proteins. CD11b⁺ cells were incubated with the indicated MIP-1α and RANTES chemokine-fusion protein variants. At different time intervals, the cells were fixed with paraformaldehyde, stained with FITC-phalloidin, and analyzed by flow cytometry. Relative F-actin was calculated as the mean fluorescence relative to that of nonstimulated cells. (C,D) In vitro chemotaxis of the murine macrophage cell line P338D1 (C) or immature bone marrow–derived DCs (D) induced by RANTES- and MIP-1α-scFv fusion proteins. Chemotactic index (± SEM of triplicate samples), defined as fold increase in the number of migrating cells in the presence of fusion proteins over spontaneous migration, is presented. Recombinant human RANTES was used as control in panels A-D. (E) Recruitment of cells into the peritoneal cavity 18 hours after intraperitoneal injection of purified chemokine-scFv fusion proteins at a 1.5 μM (chemokine) concentration. Cells/mL in 10 mL lavage fluid is given. (F) Numbers of CD11c⁺ DCs in the peritoneal cavity 18 hours after intraperitoneal injection of chemokine-scFv fusion proteins.

T cells, titrated amounts of fusion proteins were incubated with BALB/c splenocytes and Id($\lambda 2^{315}$)–specific Th2 cells that recognize aa's 91 to 101 of V_L of scFv. The T cells were derived from TCR-transgenic mice. Dimeric MIP-1α and RANTES chemokine-fusion proteins induced at least a 1000-times more efficient T-cell proliferation as measured by left shift of dose-response curves, compared with otherwise identical proteins that were unable to bind chemokine receptors due to introduced Cys-Ser mutations. The monomeric MIP-1α fusion protein was less potent than its dimeric counterparts (Figure 5A). Mouse or human dimerization units did not differ.

Chemokine-scFv plasmids injected intramuscularly induce Id priming of APCs in draining lymph nodes

To further test the T-cell activation potential of these vaccines in vivo, we injected BALB/c mice with plasmids intramuscularly, electroporated, and investigated the ability of APCs taken from draining and nondraining lymph nodes 8 days later to induce activation of $\lambda 2^{315}$-specific Th2 cells in vitro. The APCs from mice vaccinated with the dimeric humanized proteins were potent at inducing proliferation of Id-specific T cells, while APCs from mice vaccinated with the monomeric and dimeric murine MIP-1α proteins were slightly less efficient in activating T cells (Figure 5B). In vivo Id priming required an intact MIP-1α or RANTES because the Cys-Ser mutation abrogated the effect. Id priming was specific since it was abrogated when $scFv^{315}$ was exchanged with $scFv^{A20}$ in the immunizing plasmids. In vivo Id priming was observed only in draining lumbar and sacral LNs and not in nondraining LNs (data not shown).

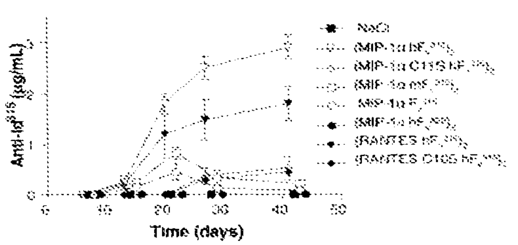

Figure 4. Antibody responses following intramuscular injection of mice with chemokine-fusion vaccine plasmids and electroporation. Serum anti-Id³¹⁵ antibodies, binding myeloma protein M315 in ELISA, at different time points after vaccination with the indicated constructs (50 μg). Error bars indicate mean plus or minus SEM.

$CD4^+$ T cells are stimulated by chemokine-fusion vaccines in vivo

Given that chemokine-scFv plasmids induced Id priming of APCs in draining LNs, we proceeded to investigate if Id-specific T-cell activation took place in vivo. Id($\lambda 2^{315}$)-specific TCR-transgenic mice on a SCID background were DNA vaccinated/electroporated and analyzed 10 days later for expression of the CD69 early activation marker on Id-specific $CD4^+$ T cells. Activation was found in the draining LNs but not in the nondraining counterparts. Activation was found with all chemokine-$scFv^{315}$ fusion proteins, and the Cys-Ser mutation abrogated activation. MIP-1α was more potent than RANTES, and human dimerization unit was more effective than mouse dimerization unit and monomers (Figure 5C).

To test if chemokine-scFv vaccines could induce in vivo proliferation of Id-specific T cells, BALB/c mice were first DNA vaccinated/electroporated, then injected with Id-specific $CD4^+$ T cells, and then given BrdU, which incorporates into dividing cells and can be detected by flow cytometry. Dimeric MIP-1α with human dimerization unit clearly induced proliferation of Id-specific $CD4^+$ T cells in draining LNs, but very little in nondraining LNs. All other chemokine-scFv induced less, but detectable, BrdU incorporation. The Cys-Ser mutation abrogated ability to induce T-cell proliferation (Figure 5D).

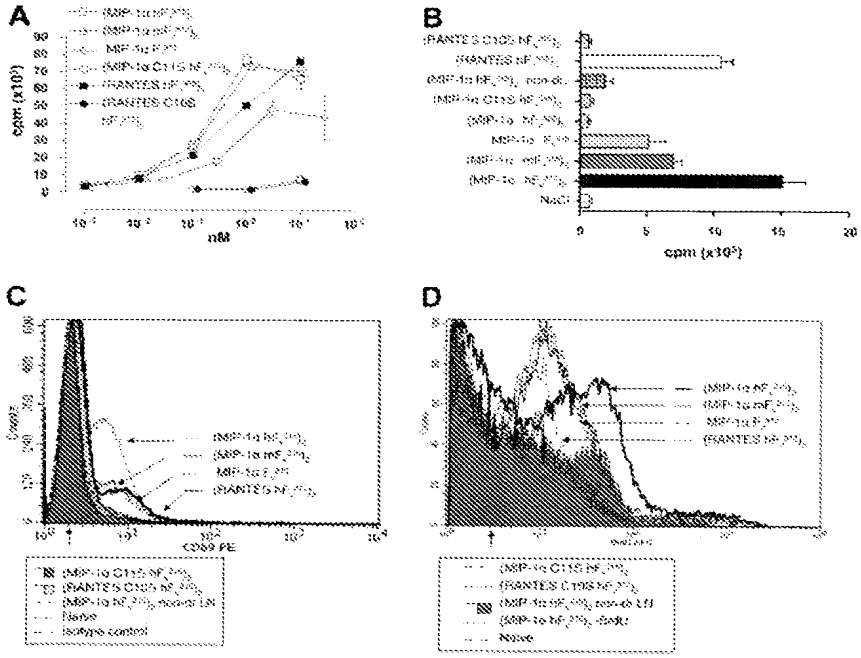

Figure 5. Chemokine-scFv fusion vaccines deliver Id to APCs for stimulation of CD4+ T cells in vitro as well as in vivo. (A) In vitro T-cell proliferation. Irradiated (8Gy) BALB/c splenocytes were incubated with titrated amounts of different purified chemokine-scFv fusion proteins (shown in nanomoles of scFv) and polarized Id($\lambda 2^{315}$)-specific Th2 cells from TCR-transgenic mice. Proliferation of T cells was measured 3 days later by incorporation of ^{3}[H] dTdR. (B) In vivo Id priming of APCs. BALB/c mice were injected in the quadriceps muscles with 50 μg of indicated plasmids, followed by electroporation. Eight days later, draining (lumbar and sacral) and nondraining LN cells were isolated, irradiated, and tested for their ability to stimulate Id($\lambda 2^{315}$)-specific 7A10B2 CD4+ T cells in a standard 3-day proliferation assay. (C) In vivo T-cell activation. Lymph node cells from vaccinated (refer to B) or naive TCR-transgenic SCID mice were isolated on day 10 and gated clonotype+ CD4+ T cells were analyzed for CD69 expression by flow cytometry. (D) In vivo T-cell proliferation. Vaccinated (refer to B) or naive BALB/c mice were reconstituted with LN and spleen cells from TCR-transgenic mice on day 7 and injected with 2 mg BrdU intraperitoneally on day 10. On day 14, gated Id-specific CD4+ T cells in LNs were analyzed for BrdU expression by flow cytometry. Error bars in panels A and B indicate means plus or minus SEM.

Chemokine-scFv DNA vaccines induce protective immunity in a myeloma and a B-cell lymphoma model

When given as a single DNA injection followed immediately by electroporation, all chemokine-scFv constructs elicited significant protective immunity against both the MOPC315 myeloma and the A20 B-cell lymphoma. A number of features should be noted. (1) The protection was Id-specific since the A20 constructs did not protect against challenges with MOPC315 and vice versa. (2) MIP-1α was more effective than RANTES. (3) Constructs with the human dimerization unit were more effective than the mouse counterpart, and monomeric constructs. (4) Introduction of the Cys-Ser mutation completely abrogated protection. Thus, the by far most effective chemokine-scFv format was dimeric, humanized MIP-1α-scFv DNA constructs that induced 70% to 80% resistance in both models, while all control mice succumbed to tumors.

CD8+ T cells are important for protection

Treatment with depleting anti-CD8 mAb significantly reduced the protection level to 20% in both tumor models (Figure 6C,D). Depleting anti-CD4 mAb reduced tumor protection by 20%, but this reduction was not significant. Anti-CD4 treatment could not be combined with MOPC315 challenge because this plasmacytoma cell line aberrantly expresses CD4 (B.B., unpublished February, 2003). We therefore used an anti–I-E^{d} mAb that blocks presentation of a well-studied Id epitope of the M315 myeloma protein (aa's

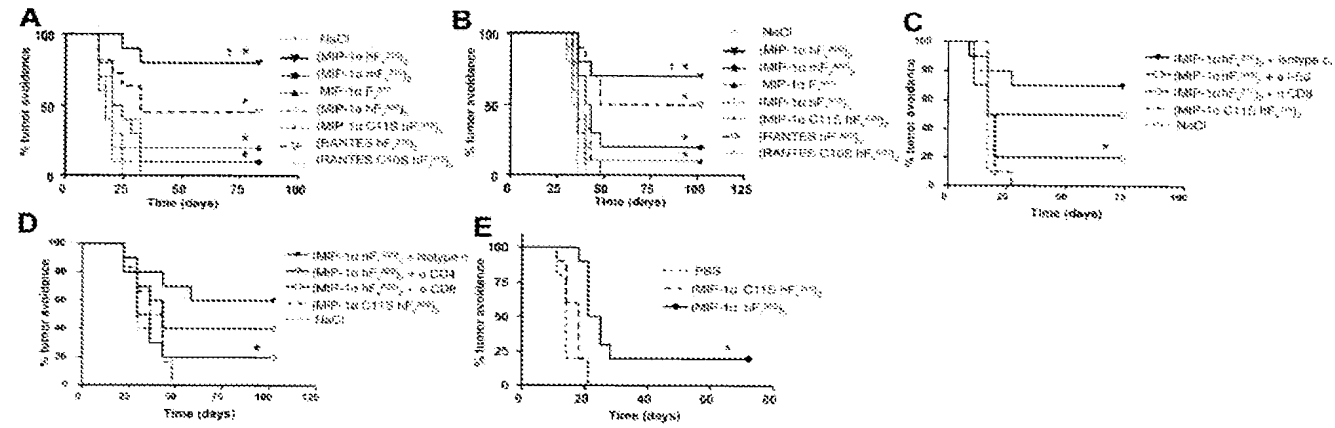

Figure 6. Chemokine-scFv DNA vaccines elicit protective immunity in a myeloma and a lymphoma model. CD8+ T cells are important for protection. BALB/c mice were injected intramuscularly with 50 μg chemokine-scFv plasmids, followed by electroporation. Fourteen days later, immunized mice were challenged with (A) 1.3×10^{5} MOPC315 myeloma cells or (B) 1.2×10^{5} A20 B cell lymphoma cells subcutaneously. A tumor of 3 mm or larger was scored as tumor take. There were 10 mice in each group. *$P \leq .05$, when compared with NaCl. †$P \leq .05$, when dimeric MIP-1α $hF_v^{315\ (or\ A20)}$ were compared with monomeric MIP-1α $F_v^{315\ (or\ A20)}$. (C,D) Requirement of T-cell subsets for protective immunity. Mice were immunized with 50 μg plasmids, repetitiously treated with anti-CD8 mAb, anti-CD4 mAb, anti–I-E^{d} mAb, or isotype-matched rat IgG from day 12, and injected with MOPC315 (C) and A20 (D) tumor cells. A significant reduction ($P \leq .05$), when compared with MIP-1α $hF_v^{315\ (or\ A20)}$ + isotype control, is marked by an asterisk. (E) BALB/c mice injected with 100 μg purified chemokine-scFv fusion proteins in PBS were injected intraperitoneally and challenged 14 days later with 1.3×10^{5} MOPC315 myeloma cells. *$P \leq .05$ compared with nontargeted chemokine-scFv^{315}.

91-101 of V_L^{315}). Such blockage has previously been shown to abrogate protection of Id-specific TCR-transgenic mice to a challenge with MOPC315.[50] In the present experiment, anti–I-E^d treatment reduced protection by 20%, but this change was not significant.

Protein vaccination is less effective than DNA vaccination/electroporation

A single subcutaneous injection of 100 μg dimeric humanized chemokine-scFv fusion proteins in PBS elicited protective immunity against a challenge with MOPC315 in 20% of the mice (Figure 6E), and was thus less effective than a single intramuscular DNA injection and electroporation. The protection depended upon functional MIP-1α.

Discussion

The results indicate that both bivalency and xenogeneic sequences in the Ig dimerization unit contributed to the enhanced activity of the dimeric chemokine-scFv fusion proteins. Thus, dimers were slightly more potent than monomers in assays that lasted only minutes to hours (induction of F-actin polymerization and chemotaxis), and much more potent in assays that lasted up to a few days (in vivo chemotaxis, in vitro T-cell proliferation). Within this time frame, adaptive immune responses directed against xenogeneic sequences should not have come into play, and especially not in vitro. Consistent with this, dimers were superior regardless of whether the dimerization motifs were of mouse (or human) origin. Possibly, dimers could bind bivalently to CCR on APCs, and thus induce better signaling. Dimers could also enhance uptake by targeted APCs, processing, and presentation of Id peptides to T cells. Supporting a potentiating role of bivalency, previous studies using monoclonal antibodies and their monovalent Fab and scFv equivalents have demonstrated that cross-linking of pre-existing chemokine receptor dimers is necessary for receptor internalization and functional responses.[25,51,52] Moreover, RANTES fused to the N-terminal end of hγ3, rendering the chemokine bivalent, induced higher levels of migration than recombinant RANTES.[53] However, the effect of cross-linking chemokine receptors has been disputed.[54] The conflicting views might be resolved if chemokine aggregation is essential only for a subgroup of chemokine receptors, or only for certain biologic functions. For example, aggregation of RANTES is essential for CCR1-mediated leukocyte arrest, while it does not affect CCR5-mediated endothelial transmigration.[55]

In immunization experiments of longer duration (Id priming of APCs and stimulation of Id-specific T cells in draining LNs, elicitation of anti-Id antibodies, and induction of Id-specific tumor resistance), the human dimerization unit was strikingly more potent than the murine counterpart. This finding is reminiscent of previous observations that xenogeneic Ig C regions enhance anti-Id antibody responses in mice.[17,56] A likely explanation in the present experiments is that the xenogeneic parts stimulate mouse $CD4^+$ T cells that deliver sustained help to anti-Id B cells.[32] Such xeno-specific $CD4^+$ T cells could, by way of T-T cooperation,[57] help Id-specific T cells crucial for B-cell tumor eradication (below). It should be stressed that these results in no way contradict a contribution of bivalency. However, bivalency in itself is clearly not sufficient. It is reasonable to suggest that bivalency and xenogeneic sequences act synergistically, but this remains to be demonstrated.

Chemokines bind different receptors variably expressed on different APCs. Thus, certain chemokines might be more efficient for targeted Id vaccination than others. In the present experiments, MIP-1α appeared more efficient than RANTES as targeting chemokine in the vaccine constructs. Mutations of the first cysteines, which destroy chemokine activity, completely abrogated responses. How well MIP-1α and RANTES compare with other chemokine-scFv proteins that have been used for Id vaccination[13-15,58] is difficult to evaluate since in the latter cases, monomeric forms were used. Also, the modes and numbers of DNA injections differed (one intramuscular injection and electroporation in our experiment vs 3 skin injections with gene gun).[13] Both laboratories have used similar numbers of A20 B lymphoma cells[13,58] for challenge of vaccinated mice, but challenge was given subcutaneously (our experiments) or intraperitoneally, and lymphoma sublines in the 2 labs might be different. Since monomeric MIP-1α $scFv^{A20}$ in the present experiments gave only about 10% protection compared with 0%, 40%, and 50% for IP-10, MCP-3, and mDF2β-$scFv^{A20}$,[13,58] respectively, it seems likely that MIP-1α and RANTES are not superior to the previously used chemokines. However, different modes of immunization do not allow firm conclusions. By contrast, the impressive 70% protection obtained with a single immunization with dimeric MIP-1α $scFv^{A20}$ only 14 days before tumor challenge surpasses previous results with Id vaccination against the A20 lymphoma based on the literature,[1,13,58] including our own with dimeric Id-fusion DNA vaccines targeted to MHC class II or CD40.[37,59] It is of interest to compare dimeric chemokine-scFv with other modes of Id vaccination. Dimeric MIP-1α $scFv^{315}$ is much more efficient than multiple immunization with myeloma protein M315, its $\lambda 2^{315}$ L-chain (30%-40% protection), and V_L fragment (no long-lasting protection), using CFA.[60]

The plasmids were injected intramuscularly combined with electroporation, a procedure that enhances transfection of muscle cells.[49,61-63] We demonstrate presence of Id-primed APCs in draining LNs, moreover, Id-specific $CD4^+$ T cells became activated and proliferated in the same LNs. These findings are similar to previous results obtained with the MHC class II– or CD40-targeted Id-fusion proteins.[37,59]

The identity of Id-primed APCs in the draining LNs is unknown. An interesting possibility is that the MHC class II^+ cells that infiltrate electroporated muscle[64] might further increase in numbers due to chemoattraction, express much of the relevant CCR (CCR1, CCR3, and CCR5), endocytose chemokine-scFv secreted by muscle cells, and migrate to draining LNs.

Targeted chemokine-scFv fusion proteins resulted in about 100 times (monomeric) to 1000 times (dimeric) better T-cell responses in vitro, compared with nontargeted, mutated versions. This is consistent with a previous study where mouse mAbs directed against CCR1, CCR2, and CCR5 on APCs were shown to cause 100 to 10 000 times better presentation of a $C\kappa^{40-48}$ peptide to DR4-restricted T cells, compared with nontargeted controls.[65] The increased presentation was shown to depend on the conventional pathway for processing of endocytosed Ag and loading of MHC class II molecules.[65] Similar results were obtained with monomeric DF2β-$scFv^{315}$ and MIP-3α-V_L^{315},[15] but in this case full titrations of targeted and nontargeted versions were not performed so that the increased efficiency of targeting CCR could be estimated only to 3 to 10 times.

Why did targeting result in better antibody responses? A plausible mechanism could be that Id-specific B cells endocytose chemokine-scFv protein, process it, and present it to T cells. Next, effector Id-specific $CD4^+$ T cells, which already have been

stimulated by Id-primed LN APCs (above), could help anti-Id–producing B cells. However, since foreign sequences greatly improved anti-Id Ab levels, it is likely that Id-specific T-cell help is of limited strength and that human IgG3-specific $CD4^+$ T cells are crucial for strong and sustained anti-Id responses.

Protein delivery of dimeric formats of the vaccine was not as efficient as intramuscular DNA vaccination and electroporation. Even so, a single injection of 100 μg protein in PBS without adjuvant induced significant protection. Obviously, protein vaccination could be optimized in several ways, such as addition of adjuvant. However, production of vaccine proteins is more laborious and expensive, compared with production of plasmids for DNA vaccination.

Even though it has been clearly demonstrated that high numbers of Id-specific $CD4^+$ T cells in TCR transgenic mice can confer tumor protection against MOPC315 in the absence of antibodies and $CD8^+$ T cells,[6,33] and even though vaccination with the $scFv^{315}$ vaccibodies clearly resulted in activation of Id-specific transgenic $CD4^+$ T cells, vaccinated BALB/c mice seem to depend predominantly on $CD8^+$ T cells for their protection against MOPC315. There has not yet been described a MHC class I–restricted T-cell epitope in M315 V regions, however, the present observations warrant a characterization of $CD8^+$ T-cell responses in the MOPC315 model. As concerns the A20 B-lymphoma model, a $CD8^+$ T-cell epitope has previously been described in the CDR3 region of V_H,[66] and $CD8^+$ T cells seem to be important in vaccinated mice in our experiments. The contribution of Id-specific $CD8^+$ T cells require that the chemokine-fusion molecules get access to the MHC class I processing and presentation pathway. Supporting this, it has recently been described that antigens linked to MIP-3α are endocytosed, gain access to the cytosol, and are presented on MHC class I molecules in a TAP-dependent fashion.[67,68]

Targeting antigen to chemokine receptors should be further investigated for their ability to induce cross-presentation on MHC class I molecules and $CD8^+$ T-cell responses. The possibility that the dimeric chemokine format enhances cross-presentation should be considered.

Acknowledgments

The work was supported by the Norwegian Cancer Society, the Research Council of Norway, Medinnova SF, and the Multiple Myeloma Research Foundation.

We would like to thank Inovio, Oslo, for providing the electroporator. The technical help of Hilde Omholt, Tuva H. Hereng, Mona M. Lindeberg, Tom-Ole Løvås, and Beate Hegge is gratefully acknowledged. Drs Keith Thompson and Pier Ruffini critically read the paper.

Authorship

Contribution: B.B. participated in designing the research and writing the paper; A.B.F. performed the experiments, analyzed data, and participated in designing the research and writing the paper.

Conflict-of-interest disclosure: Both authors (A.B.F. and B.B.) hold a patent pending related to the work that is described in the present study.

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